The interactions with Ro60 and La differentially affect nuclear export of hY1 RNA

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ABSTRACT

Ro RNPs are evolutionarily conserved ribonucleoprotein particles that consist of a small RNA, known as Y RNA, associated with several proteins, such as La, Ro60, and Ro52. The Y RNAs (Y1–Y5), which are transcribed by RNA polymerase III, have been shown to reside almost exclusively in the cytoplasm as Ro RNPs. To obtain more insight into the nuclear export pathway of Y RNAs, hY1 RNA export was studied in *Xenopus laevis* oocytes. Injection of various hY1 RNA mutants showed that an intact Ro60 binding site is a prerequisite for nuclear export, whereas the presence of an intact La binding site resulted in strong nuclear retention of hY1 RNA. Competition studies with various classes of RNAs indicated that, in addition to Ro60, another titratable factor was necessary for nuclear export of hY1 RNA. This factor appears also to be involved in nuclear export of tRNA. Because export of hY1 RNA could not be blocked by a synthetic peptide containing the recently identified nuclear export signal of the HIV-1 Rev protein, nuclear export of hY1 RNA does not seem to be dependent on a Rev-like nuclear export signal.

Keywords: La (SS-B); nuclear export; Ro RNP; Ro (SS-A); Y RNA

INTRODUCTION

Ro RNPs are ribonucleoprotein particles that consist of a Y RNA molecule and several proteins (reviewed in Van Venrooij et al., 1993). Four different RNA polymerase III-transcribed Y RNAs have been characterized in human cells. They vary in length from 84 to 112 nt, and are referred to as: hY1, hY3, hY4, and hY5 RNA (Hendrick et al., 1981). The secondary structure of these homologous Y RNAs is characterized by base pairing of the conserved 3'- and 5'-ends of the RNA molecules (Wolin & Steitz, 1983; Van Gelder et al., 1994). Two common proteins have been shown to bind directly to the Y RNAs: Ro60, which binds to the terminal part of the conserved stem structure (stem 1, see Fig. 1) (Wolin & Steitz, 1984; Pruijn et al., 1991), and La, which binds to the 3' oligo-uridylate stretch of the RNA molecules (Mathews & Francoeur, 1984; Stefano, 1984; Pruijn et al., 1991). Ro52, another putative Ro RNP protein, does not bind directly to the Y RNAs, but probably associates with the particle via protein–protein interaction (Slobbe et al., 1992).

The Y RNAs (Hendrick et al., 1981; O'Brien et al., 1993; Pruijn et al., 1993; Farris et al., 1995; Van Horn et al., 1995), the Ro60 protein (Chan & Buyon, 1994 and references therein; Van Horn et al., 1995), and the La protein (Pruijn, 1994 and references therein) of the Ro RNPs are well conserved during evolution. Using a variety of techniques, such as enucleation of tissue culture cells (O'Brien et al., 1993; Peek et al., 1993) and injection of *Xenopus laevis* oocytes (O'Brien et al., 1993; Simons et al., 1994), it has been shown that Ro RNPs are located in the cytoplasm. As a consequence, newly RNA polymerase III-transcribed Y RNAs have to be exported to the cytoplasm.

Very little is known about the mechanism of nuclear export of RNA (reviewed in Izaurralde & Mattaj, 1995; Simos & Hurt, 1995). Analogous to the mechanism of nuclear import, it is to be expected that export involves binding of specific proteins to RNAs that have to be exported. Consistent with this prediction, nuclear export of several RNAs has been shown to be a saturable, energy requiring, carrier-mediated process. (Zasloff, 1983; Hamm & Mattaj, 1990; Jarmolowski et al., 1994).

Much of the information available on RNA export concerns RNA polymerase II transcripts such as mRNA and U1 snRNA. In the nucleus, mRNA is associated

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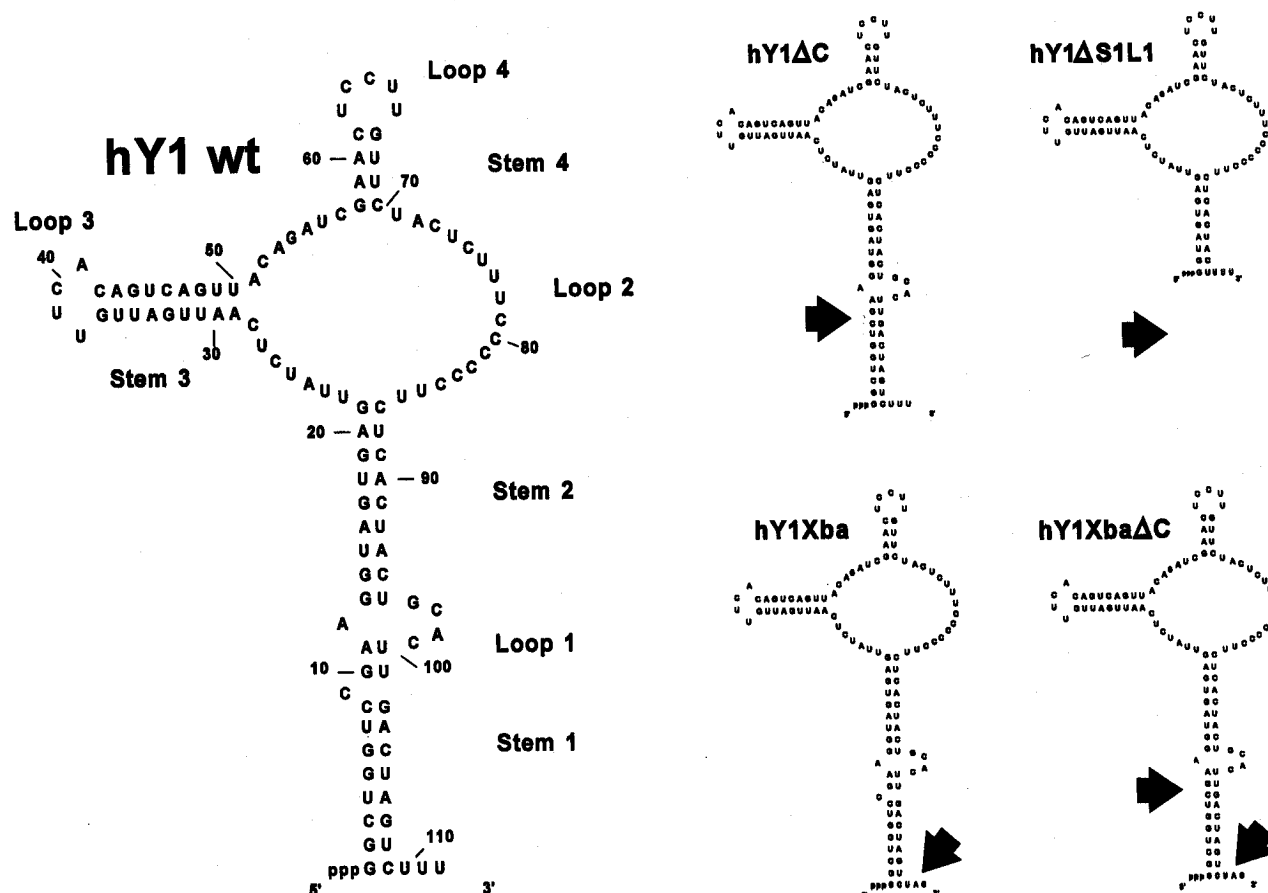
FIGURE 1. Secondary structure models for hY1wt RNA and mutants. The structure of hY1wt RNA was proposed by Van Gelder et al. (1994), and the structures of the mutants were based upon this hY1wt RNA structure. Arrows indicate positions of the mutations.

with the very abundant heterogeneous nuclear RNP (hnRNP) proteins. Several of these proteins shuttle between nucleus and cytoplasm and, for hnRNP A1, it has been shown that it was associated with poly(A)+ RNA in both compartments, suggesting that hnRNP A1 mediates this export (Pinol-Roma & Dreyfuss, 1991, 1992, 1993). Processing steps leading to the formation of the 3'-end of mRNA in some way appeared to be coupled to export of mRNA (Eckner et al., 1991). The mono-methyl guanosine cap structure, present at the 5'-end of all RNA polymerase II transcripts, has been shown to be important for the export of both U snRNAs and mRNAs (Hamm & Mattaj, 1990; Jarmolowski et al., 1994). A nuclear cap binding protein (CBP) has been identified and has been shown to be involved in export of these RNAs (Izaurralde et al., 1994).

For the RNA polymerase III product 5S rRNA it has been shown that mutants that could not bind to L5 or TFIIIA were not exported (Guddat et al., 1990), suggesting a role for these proteins in the export process. Via a similar method, a role for SRP9/SRP14 in the export of SRP RNA (He et al., 1994) was established.

In the study reported here, we investigated the export mechanism of Y RNAs. Previously, we showed that hY RNAs after injection into the nucleus of *X. laevis* oocytes are exported to the cytoplasm in an energy-dependent way (Simons et al., 1994). Now we extend these studies and show that the Ro60 protein is involved in hY1 RNA export, in contrast to the La protein, binding of which leads to an opposite effect, that is, nuclear retention of Y RNAs. Furthermore, we present evidence that the export of Ro RNPs is a saturable process and that, in addition to Ro60, another *trans*-acting factor is involved. This factor is also involved in the export of tRNA.

RESULTS

### Role of Ro60 and La in the export of hY1 RNA

Previous experiments have shown that in vitro transcribed hY RNAs accumulate in the cytoplasm of *X. laevis* oocytes in an energy-dependent way (O'Brien et al., 1993; Simons et al., 1994). As has been shown

for nuclear export of other RNAs (Izaurralde & Mattaj, 1995; Simos & Hurt, 1995), it seems likely that export of hY RNAs is also mediated by specific nuclear proteins. For two proteins, i.e., Ro60 and La, it has been shown that they bind in the nucleus to hY RNAs (Simons et al., 1994). To investigate whether Ro60 and La are involved in nuclear export of hY1 RNA, mutant hY1 RNAs were constructed, in which the binding sites for these proteins were either changed or deleted.

The different hY1 RNA mutants, used for export studies, are depicted in Figure 1. To interfere with the binding of Ro60 to hY1 RNA, the terminal part of the conserved stem (stem 1), which is the binding site for Ro60 (Wolin & Steitz, 1984; Pruijn et al., 1991), had to be changed. In hY1ΔC RNA, the bulged C at position 9 in stem 1 is deleted. An equivalent deletion in hY5 RNA resulted in reduced binding of human Ro60 in vitro (Pruijn et al., 1991). Also, in the case of hY1ΔC RNA, the deletion resulted in a lower affinity in vitro for Ro60 as compared to wild-type hY1 RNA (Fig. 2, compare lane 12 with lane 11). In a second mutant (hY1ΔS1L1), the complete terminal part and the internal loop of the conserved stem structure (stem 1 and loop 1) are deleted. This mutant is therefore unable to associate with Ro60 (Fig. 2, lane 15). Because both mutants still do contain the 3′ oligo uridine stretch, which is the primary binding site for the La protein (Mathews & Francoeur, 1984; Stefano, 1984; Pruijn et al., 1991), both are able to bind the La protein (Fig. 2, lanes 7, 10).

After in vitro transcription of hY1wt, hY1ΔC, and hY1ΔS1L1 DNA, the resulting ^{32}P-labeled RNAs were injected into the nucleus of oocytes. At various time points after injection, the oocytes were dissected and

the distribution between nucleus and cytoplasm was examined. In vitro transcribed U1 snRNA with a mutated Sm-binding site (U1ΔSm), which is known to leave the nucleus and to remain in the cytoplasm (Hamm & Mattaj, 1990), was co-injected as an internal control. In Figure 3A, it can be seen that hY1wt RNA was exported gradually to the cytoplasm, as was observed before (Simons et al., 1994). Complete export, however, takes more than 8 h. Also hY1ΔC RNA was exported to the cytoplasm, but with a much lower efficiency (Fig. 3B). Eight hours after injection, only a minor part of hY1ΔC RNA is present in the cytoplasm (Fig. 3B, lanes 8, 9), whereas at that time point already half of the amount of hY1wt RNA has been exported to the cytoplasm (Fig. 3A, lanes 8, 9). The lower rate of export of hY1ΔC RNA is most probably caused by the decreased affinity of hY1ΔC RNA for Ro60 in comparison with hY1wt RNA. A more drastically reduced export rate is observed for hY1ΔS1L1 RNA. In this case, absolutely no export could be detected (Fig. 3C). After prolonged exposure of the autoradiograph, it became evident that, even after 24 h, no export of hY1ΔS1L1 RNA could be observed. In contrast to this, mutants hY1ΔS2L1 (in which stem 2 and loop 1 are deleted), hY1ΔS3L3 (in which stem 3 and loop 3 are deleted), and hY1ΔS4L4 (in which stem 4 and loop 4 are deleted) were exported to the cytoplasm with efficiencies similar to hY1wt (data not shown). In view of the fact that hY1ΔS1L1 RNA does not associate detectably with Ro60, these results suggest strongly that Ro60 binding is necessary for the export of hY1 RNA. Note that both hY1ΔC and hY1ΔS1L1 seem to be somewhat less stable than wild-type hY1 RNA (compare Fig. 3A
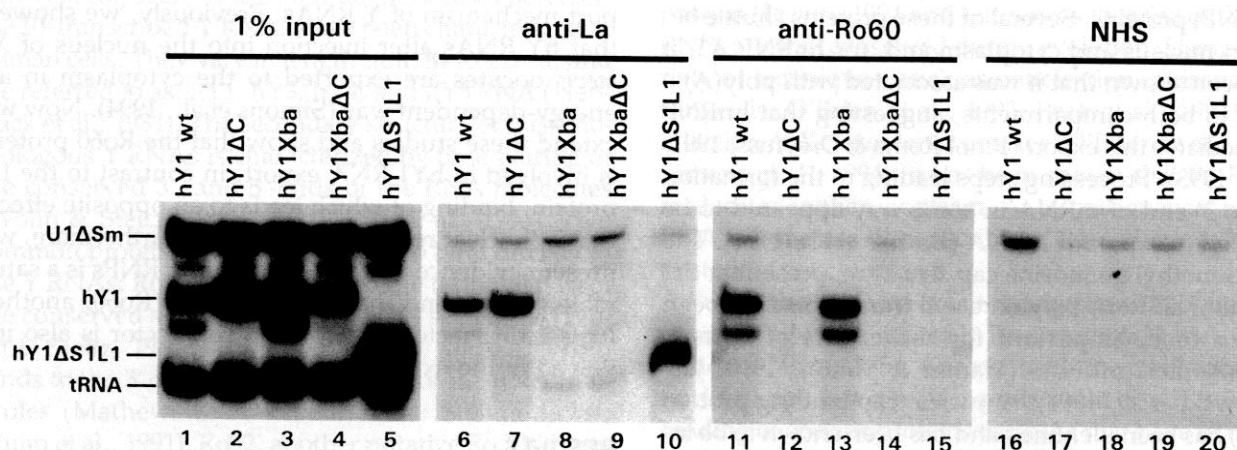
FIGURE 2. Ability of mutant hY1 RNAs to bind La and Ro60 proteins. In vitro transcribed hY1 RNA, U1ΔSm snRNA, and tRNA$_i^{met}$ were incubated in HeLa S100 extract and subsequently precipitated with monoclonal anti-La antibodies (lanes 6–10), monoclonal anti-Ro60 antibodies (lanes 11–15), or normal human serum (NHS) (lanes 16–20). RNA was isolated from the precipitates (lanes 6–20) or from 1% of the input RNA mixture (lanes 1–5) and analyzed by denaturing gel electrophoresis. hY1wt, hY1ΔC, hY1Xba, and hY1XbaΔC migrate at approximately the same position in the gel, indicated by hY1. The relatively efficient precipitation of hY1wt RNA with NHS (lane 16) in this particular experiment appeared to be irreproducible. Note that, due to a more efficient precipitation, the anti-La samples (lanes 6–10) are autoradiographed much shorter than the other samples.
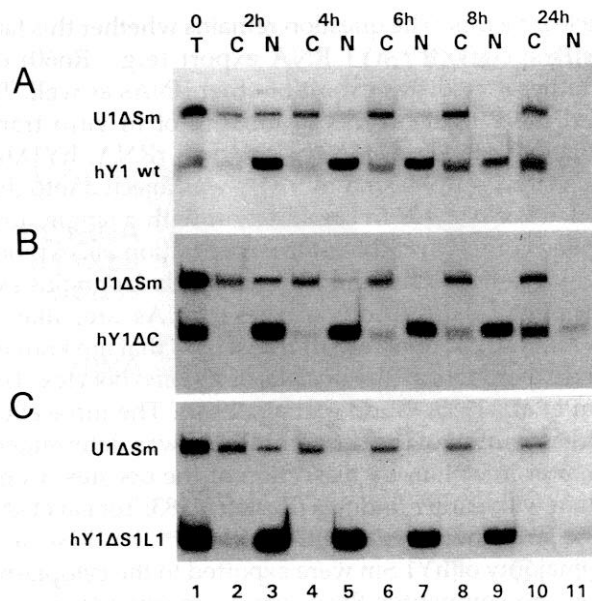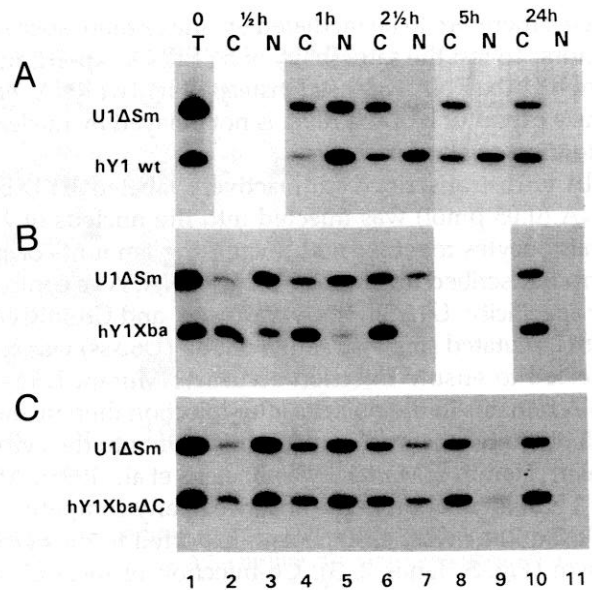
FIGURE 3. Nuclear export of hY1 RNA mutants defective in Ro60 binding. In vitro transcribed U1ΔSm RNA was co-injected with either (**A**) hY1wt RNA, (**B**) hY1ΔC RNA, or (**C**) hY1ΔS1L1 RNA into *X. laevis* oocyte nuclei. RNA was extracted from total oocytes (T), cytoplasmic (C), or nuclear (N) fractions immediately (lane 1), 2 h (lanes 2, 3), 4 h (lanes 4, 5), 6 h (lanes 6, 7), 8 h (lanes 8, 9), or 24 h (lanes 10, 11) after injection and analyzed on a denaturing polyacrylamide gel.

FIGURE 4. Nuclear export of hY1 RNA mutants defective in La binding. In vitro transcribed U1ΔSm RNA was co-injected with either (**A**) hY1wt RNA, (**B**) hY1Xba RNA, or (**C**) hY1XbaΔC RNA into *X. laevis* oocyte nuclei. RNA was extracted from total oocytes (T), cytoplasmic (C), or nuclear (N) fractions immediately (lane 1), 0.5 h (lanes 2, 3), 1 h (lanes 4, 5), 2.5 h (lanes 6, 7), 5 h (lanes 8, 9), or 24 h (lanes 10, 11) after injection or analyzed on a denaturing polyacrylamide gel.

with Fig. 3B,C). This suggests that binding of Ro60 might stabilize the hY1wt RNA. Control injections of these RNAs into the cytoplasm revealed that they are stable in the cytoplasmic compartment during the coarse of the experiment, implying that degradation indeed occurs in the nucleus (data not shown).

To analyze the influence of La binding on the export of hY1 RNA, two hY1 RNA mutants were made (hY1Xba and hY1XbaΔC) in which the binding site of La on hY1 RNA was destroyed by mutating the 3' terminal UUU sequence into UAG (Fig. 1). In vitro binding experiments showed that both RNAs are indeed unable to bind the La protein (Fig. 2, lanes 8, 9). In addition to the mutation at the 3' end of both molecules, one of these mutants (hY1XbaΔC) also lacks the bulged C at position 9 as in hY1ΔC RNA. This means that hY1XbaΔC RNA not only is unable to interact with La, but that it has a decreased binding affinity for Ro60 as well (Fig. 2, compare lane 13 with 14).

Figure 4 shows the results of injection of these radio-labeled RNAs into the nucleus of *X. laevis* oocytes. Mutant hY1Xba RNA (Fig. 4B) was, like hY1wt RNA (Fig. 4A), transported to the cytoplasm, indicating that La binding is not necessary for export of hY1 RNA from the nucleus. On the contrary, the inability of hY1Xba RNA to interact with La appeared to increase the export efficiency dramatically. One hour after injection, the majority of hY1wt RNA is still in the nucleus (Fig. 4A, lanes 4, 5), whereas hY1Xba RNA has almost

completely been transported to the cytoplasm (Fig. 4B, lanes 4, 5). The most likely explanation for this result is that binding of hY1 RNA to the La protein retains the hY1 RNA in the nucleus of *X. laevis* oocytes. This is further substantiated by the fact that nuclear hY1wt RNA still contains the La binding site, whereas the cytoplasmic hY1 RNA in the oocyte is shortened at the 3' end by 2–3 nt, which disrupts the La binding site (data not shown).

To investigate whether an intact Ro60 binding site is also necessary for the export of the hY1Xba RNA, hY1XbaΔC RNA was tested for its ability to leave the nucleus. Indeed, mutant hY1XbaΔC RNA was able to leave the nucleus (Fig. 4C), albeit with a lower efficiency than observed for hY1Xba RNA. Although hY1Xba RNA is almost completely exported to the cytoplasm within 1 h (Fig. 4B, lanes 4, 5), the hY1XbaΔC RNA reached a similar distribution in about 5 h (Fig. 4C, lanes 8, 9). Thus, as observed for export of hY1wt RNA, efficient export of hY1Xba RNA also seems to be dependent on the binding of Ro60.

Saturation of hY1 RNA export

Previously it was shown that, in competition experiments with tRNA, U1 snRNA, and mRNA, increasing amounts of any of these RNAs specifically saturated their own export, whereas cross-competition with other RNAs had little or no effect (Jarmolowski et al., 1994). At least one step in the export of these RNAs

seems therefore to be mediated by one or more specific factors. To test the saturability of hY1 RNA export, mutant hY1Xba RNA was used instead of hY1wt RNA, because export of hY1Xba RNA is not delayed by nuclear retention as shown above.

In vitro transcribed radioactively labeled hY1Xba RNA (0.05 pmol) was injected into the nucleus of *X. laevis* oocytes together with increasing amounts of in vitro transcribed unlabeled hY1Xba RNA. As a control for specificity, U1ΔSm RNA was used, and U6 snRNA with a mutated single-stranded region (U6Δss) was co-injected to ensure injection accuracy. Mutant U6Δss RNA remains in the nucleus after injection therein and will not enter the nucleus after injection into the cytoplasm (Hamm & Mattaj, 1989; Boelens et al., 1995). After 1 h of incubation in the absence of any competitor, hY1Xba RNA was, as expected, exported to the cytoplasm (Fig. 5, lanes 2, 3). Co-injection of increasing amounts of unlabeled competitor hY1Xba RNA (0.21–2.5 pmol per oocyte) resulted in progressive inhibition of export, confirming that the export process was saturable (Fig. 5). With 2.5 pmol of competitor, export of labeled hY1Xba RNA was completely blocked (Fig. 5, lanes 10, 11). The competition with hY1Xba RNA appears to be specific, because the export of U1ΔSm RNA was not influenced. U6Δss RNA was, as expected, not transported at all. When hY1Xba RNA as competitor was replaced by hY1wt RNA, the latter RNA appeared to compete as good as the hY1Xba RNA mutant (data not shown).

Competition for a common hY1 RNA export factor

The saturation experiments indicate that nuclear export of hY1Xba RNA is mediated by one or more limiting

nuclear factors. The question remains whether this factor is specific for hY1 RNA export (e.g., Ro60) or whether it mediates export of other RNAs as well. To shed more light on this, a mixture of in vitro transcribed ^{32}P-labeled U1ΔSm RNA, 5S rRNA, hY1Xba RNA, U6Δss RNA, and tRNA$_i^{met}$ was injected into the nucleus of oocytes in combination with a single, unlabeled competitor RNA at a concentration of 2.5 pmol per oocyte. 5S rRNA and tRNA are included in this experiment because both of these RNAs are, like Y RNAs, RNA polymerase III transcripts that are known to be exported from the nucleus of *X. laevis* oocytes (Tobian et al., 1985; Guddat et al., 1990). The intracellular distribution of the different RNAs was determined 3 h after injection, by dissection of the oocytes. Consistent with earlier findings (Zasloff, 1983; Tobian et al., 1985; Jarmolowski et al., 1994), tRNA$_i^{met}$, hY1Xba, and the majority of hY1 Sm were exported to the cytoplasm when no competitor RNA was co-injected (Fig. 6A, lanes 2, 3). In contrast, both 5S rRNA and U6Δss RNA were not detectably exported within 3 h after injection (Fig. 6A, lanes 2, 3).

When 2.5 pmol of competitor hY1Xba RNA was co-injected with the mixture, only the export of hY1Xba RNA was blocked, whereas the export of U1ΔSm RNA and tRNA$_i^{met}$ was not influenced (Fig. 6A, lanes 4, 5), suggesting that the limiting nuclear export factor of hY1Xba RNA is specific for this type of RNA. Similarly, export of neither hY1Xba RNA nor tRNA$_i^{met}$ was hindered by competition with U1ΔSm RNA, whereas the export of U1ΔSm RNA was almost completely inhibited under these circumstances (Fig. 6A, lanes 8, 9). Thus, the export pathway of hY1 RNA is distinct from that followed by U1 snRNA. Surprisingly, 2.5 pmol of tRNA$_i^{met}$ not only competed for tRNA$_i^{met}$ export as expected, but also severely interfered with the export of hY1Xba RNA to the cytoplasm (Fig. 6A, lanes 6, 7). This inhibition of hY1Xba RNA export by an excess of tRNA$_i^{met}$ seems to be in contradiction with the result that export of tRNA$_i^{met}$ could not be blocked by co-injection of 2.5 pmol of hY1Xba RNA. However, co-injections of larger amounts of competitor hY1Xba RNA (up to 5 pmol) did result in significant inhibition of tRNA$_i^{met}$ export when analyzed 1 h after injection (Fig. 6B, compare lanes 2, 3 with 4, 5), suggesting strongly that export of hY1Xba RNA and tRNA$_i^{met}$ is dependent on a common factor.

The binding of the latter factor to hY1 RNA could either be Ro60-dependent, meaning that it requires the Ro60 protein for association with the hY1 RNA, or Ro60-independent. To investigate this, the competition experiment was performed with hY1ΔS1L1 RNA as competitor, i.e., the mutant that is unable to interact with Ro60. As can be seen in Figure 6C, hY1ΔS1L1 RNA competed equally well for export of hY1Xba RNA (lanes 6, 7) as hY1Xba itself (lanes 4, 5). In combination with the observation that hY1ΔS1L1 RNA also com-
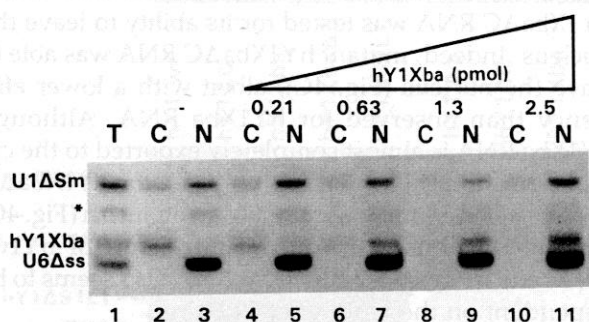
FIGURE 5. Saturability of hY1 RNA export. A mixture of in vitro transcribed, ^{32}P-labeled hY1Xba RNA (0.05 pmol), U1ΔSm RNA, and U6Δss RNA was co-injected into *X. laevis* oocyte nuclei without (lanes 1–3) or together with 0.21 pmol (lanes 4, 5), 0.63 pmol (lanes 6, 7), 1.3 pmol (lanes 8, 9), or 2.5 pmol (lanes 10, 11) nonlabeled hY1Xba RNA. RNA was extracted from total oocytes (T; 2 oocyte equivalents), cytoplasmic (C; 4 oocyte equivalents), or nuclear (N; 4 oocyte equivalents) fractions immediately (lane 1) and 1 h (lanes 2–11) after injection and analyzed on a denaturing polyacrylamide gel. The band migrating at the position marked with an asterisk is a U6Δss RNA-related product.
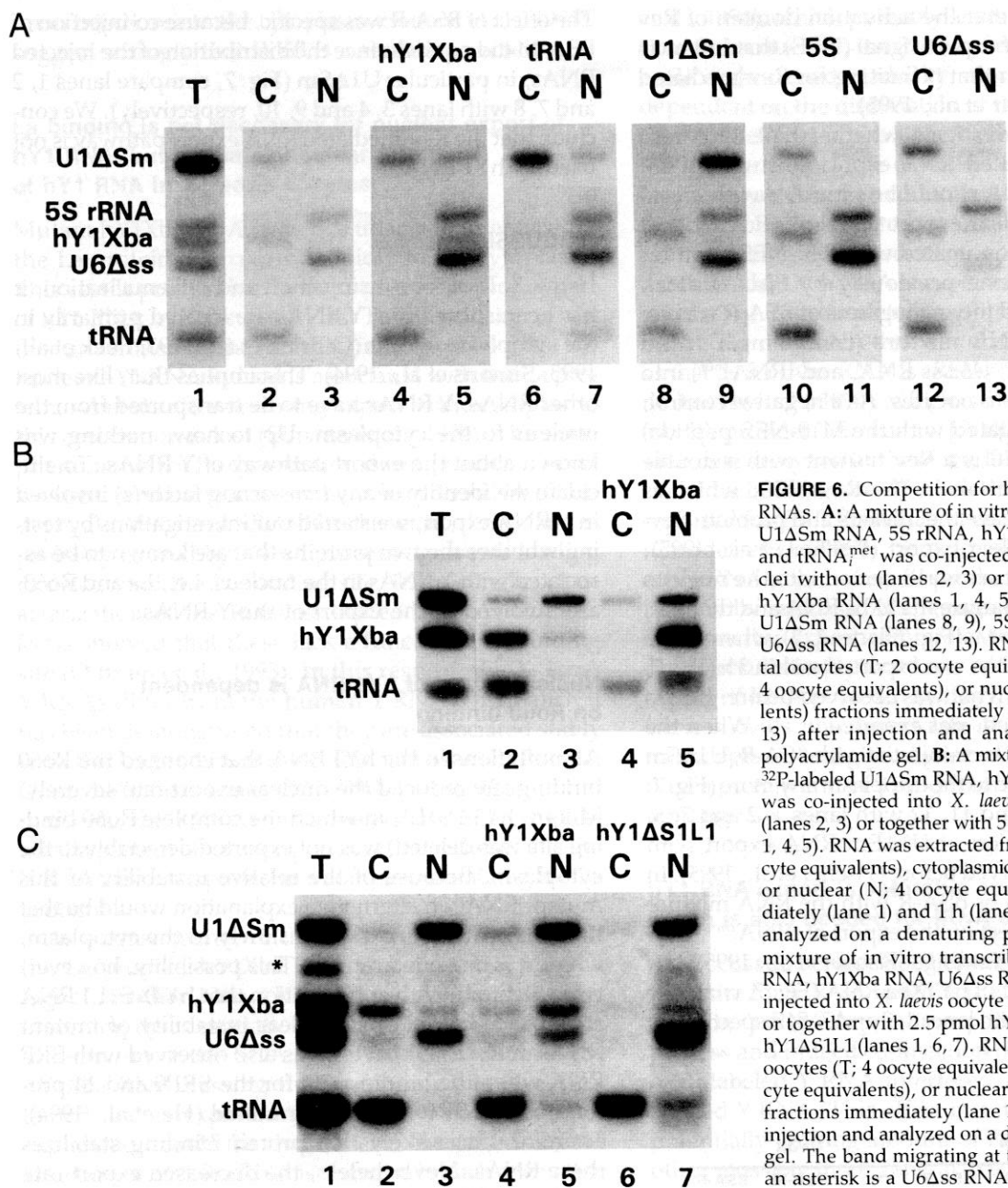
FIGURE 6. Competition for hY1 RNA export by other RNAs. **A:** A mixture of in vitro transcribed, ^{32}P-labeled U1ΔSm RNA, 5S rRNA, hY1Xba RNA, U6Δss RNA, and tRNA$_i^{met}$ was co-injected into *X. laevis* oocyte nuclei without (lanes 2, 3) or together with 2.5 pmol hY1Xba RNA (lanes 1, 4, 5), tRNA$_i^{met}$ (lanes 6, 7), U1ΔSm RNA (lanes 8, 9), 5S rRNA (lanes 10, 11), or U6Δss RNA (lanes 12, 13). RNA was extracted from total oocytes (T; 2 oocyte equivalents), cytoplasmic (C; 4 oocyte equivalents), or nuclear (N; 4 oocyte equivalents) fractions immediately (lane 1) or 3 h (lanes 2–13) after injection and analyzed on a denaturing polyacrylamide gel. **B:** A mixture of in vitro transcribed ^{32}P-labeled U1ΔSm RNA, hY1Xba RNA, and tRNA$_i^{met}$ was co-injected into *X. laevis* oocyte nuclei without (lanes 2, 3) or together with 5 pmol hY1Xba RNA (lanes 1, 4, 5). RNA was extracted from total oocytes (T; 2 oocyte equivalents), cytoplasmic (C; 4 oocyte equivalents), or nuclear (N; 4 oocyte equivalents) fractions immediately (lane 1) and 1 h (lanes 2–5) after injection and analyzed on a denaturing polyacrylamide gel. **C:** A mixture of in vitro transcribed, ^{32}P-labeled U1ΔSm RNA, hY1Xba RNA, U6Δss RNA, and tRNA$_i^{met}$ was co-injected into *X. laevis* oocyte nuclei without (lanes 2, 3) or together with 2.5 pmol hY1Xba RNA (lanes 4, 5) or hY1ΔS1L1 (lanes 1, 6, 7). RNA was extracted from total oocytes (T; 4 oocyte equivalents), cytoplasmic (C; 4 oocyte equivalents), or nuclear (N; 4 oocyte equivalents) fractions immediately (lane 1) and 1 h (lanes 2–7) after injection and analyzed on a denaturing polyacrylamide gel. The band migrating at the position marked with an asterisk is a U6Δss RNA-related product.

peted for tRNA$_i^{met}$ export (Fig. 6C, lanes 6, 7), these results suggest strongly that the common export factor acts independent of Ro60.

The question remains whether other RNA polymerase III products, such as 5S rRNA and U6 snRNA, are also able to compete for the export of hY1Xba RNA. Previously, it has been shown that both 5S rRNA and U6 snRNA are able to leave the nucleus of *X. laevis* oocytes (Guddat et al., 1990; Boelens et al., 1995). Therefore, the competition experiments were performed with either an excess of 5S rRNA (Fig. 6A, lanes 10, 11) or U6Δss RNA (Fig. 6A, lanes 12, 13). In both cases, no interference with hY1 RNA (or tRNA$_i^{met}$ or U1 snRNA) export could be observed.

In conclusion, export of hY1 RNA is mediated by a nuclear factor, which is also necessary for the export of tRNA$_i^{met}$. This factor is probably not shared by other RNA polymerase III transcripts such as 5S rRNA and U6 snRNA, and is not involved in the export process of U1 snRNA.

Competition of hY1 RNA export with the Rev-NES

The best-studied protein involved in RNA export is Rev, a human immunodeficiency virus protein, which binds to the Rev response element (RRE) present in unspliced viral RNAs, resulting in nuclear export of these unspliced viral RNAs (Fischer et al., 1994). Recently,

it has been shown that the activation domain of Rev represents a nuclear export signal (NES) that interacts with a cellular factor that is limiting for Rev-mediated RNA export (Fischer et al., 1995).

We wanted to investigate whether hY1 RNA also uses the Rev-mediated RNA export pathway. If so, transport of hY1 RNA should be saturable with an excess of the Rev nuclear export signal peptide. To test this, we used BSA conjugated with Rev-NES peptides (BSA-R), as was done previously by Fischer et al. (1995). Two-hundred fifty nanograms of BSA-R was co-injected with an RNA mixture (containing U1ΔSm RNA, hY1Xba RNA, U6Δss RNA, and tRNA$_i^{met}$) into the nucleus of *X. laevis* oocytes. As a negative control, BSA-M (BSA conjugated with the M10-NES peptide) was co-injected. M10 is a Rev mutant with a double amino acid substitution in the Rev-NES, which is therefore unable to leave the nucleus and promote Rev-mediated RNA nuclear export (Fischer et al., 1995). When the RNA mixture was injected into the nucleus without any BSA conjugate, hY1Xba RNA and tRNA$_i^{met}$ were exported within 1 h (Fig. 7, lanes 1, 2), whereas the majority of U1ΔSm RNA was exported within 3 h (Fig. 7, lanes 7, 8), similar to what was observed before. U6Δss RNA was, as expected, not exported at all. When the same RNA mixture was co-injected with BSA-R, U1ΔSm RNA was not exported to the cytoplasm any more (Fig. 7, compare lanes 5, 6 and 11, 12 with lanes 1, 2 and 7, 8, respectively). Inhibition of U1ΔSm RNA export with BSA-R has been observed before (Fischer et al., 1995). In contrast, co-injection of BSA-R with the RNA mixture had no noticeable effect on the export of tRNA$_i^{met}$, consistent with previous findings (Fischer et al., 1995), but also not on the export of hY1Xba RNA (Fig. 7, compare lanes 5, 6 and 11, 12 with lanes 1, 2 and 7, 8, respectively).

The effect of BSA-R was specific, because co-injection of BSA-M did not influence the distribution of the injected RNAs, in particular U1ΔSm (Fig. 7, compare lanes 1, 2 and 7, 8 with lanes 3, 4 and 9, 10, respectively). We conclude that the Rev-mediated RNA export pathway is not used by hY1 RNA.

DISCUSSION

Using *X. laevis* oocyte injection and cell enucleation, it has been shown that Y RNAs are located primarily in the cytoplasm of cells (O'Brien et al., 1993; Peek et al., 1993; Simons et al., 1994). This implies that, like most other RNAs, Y RNAs have to be transported from the nucleus to the cytoplasm. Up to now, nothing was known about the export pathway of Y RNAs. To elucidate the identity of any *trans*-acting factor(s) involved in Y RNA export, we started our investigations by testing whether the two proteins that are known to be associated with Y RNAs in the nucleus, i.e., La and Ro60, are involved in the export of the Y RNAs.

Nuclear export of hY1 RNA is dependent on Ro60 binding

All mutations in the hY1 RNA that changed the Ro60 binding site reduced the nuclear export rate severely. Mutant hY1ΔS1L1, in which the complete Ro60 binding site was deleted, was not exported detectably to the cytoplasm. Because of the relative instability of this mutant RNA, an alternative explanation would be that hY1ΔS1L1 RNA is exported slowly to the cytoplasm, where it is degraded rapidly. This possibility, however, was ruled out by the observation that hY1ΔS1L1 RNA is stable in cytoplasm. Nuclear instability of mutant RNAs in *X. laevis* oocytes was also observed with SRP RNA when the binding site for the SRP9 and 14 proteins on the SRP RNA was mutated (He et al., 1994). Therefore, it is likely that protein binding stabilizes these RNAs. Nevertheless, the decreased export rate of hY1ΔC RNA and the lack of export of hY1ΔS1L1 RNA are most likely explained by assuming that Ro60 binding is necessary for export of hY1 RNA. This conclusion is in good agreement with our previous results, which suggested that Ro60 associates with hY1 RNA in the nucleus and that, after this association, the RNP is exported rapidly to the cytoplasm (Simons et al., 1994). Furthermore, it has been shown that Ro60 is both present in the cytoplasm and in the nuclei of *X. laevis* oocytes (Simons et al., 1994), human (Peek et al., 1993), and mouse cells (O'Brien et al., 1993). Further studies are needed to find (1) possible factor(s) to which the Ro60 protein binds in order to activate nuclear export and (2) which part of Ro60 is involved in such an interaction. The finding that Ro60 is able to leave the nucleus associated with the Y RNAs, and the fact that Ro60 is actively transported to the nucleus
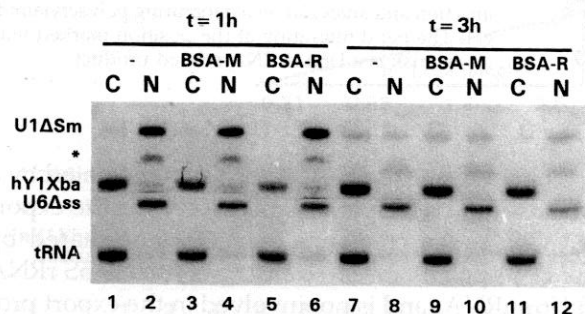
FIGURE 7. Competition for hY1 RNA export with BSA-Rev NES conjugates. A mixture of in vitro transcribed ^{32}P-labeled U1ΔSm RNA, hY1Xba RNA, U6Δss RNA, and tRNA$_i^{met}$ was co-injected into *X. laevis* oocyte nuclei together with 0.25 μg BSA-R (BSA conjugated with Rev-NES peptides) (lanes 5, 6 and 11, 12), 0.25 μg BSA-M (BSA conjugated with mutant Rev-NES peptides) (lanes 3, 4, and 9, 10), or without competitor protein (lanes 1, 2 and 7, 8). RNA was extracted from cytoplasmic (C) and nuclear (N) fractions 1 h (lanes 1–6) and 3 h (lanes 7–12) after injection and analyzed on a denaturing polyacrylamide gel. The band migrating at the position marked with an asterisk is a U6Δss RNA-related product.

upon injection into the cytoplasm (Simons et al., 1994), suggest strongly that Ro60 is a shuttling protein.

La binding is not necessary for nuclear export of hY1 RNA, but mediates nuclear retention of hY1 RNA in *X. laevis* oocytes

Mutant hY1Xba RNA, which is unable to interact with the La protein, is exported rapidly to the cytoplasm. This result proves that La is not necessary for export of hY1 RNA in *X. laevis* oocytes. This conclusion is further underlined by the fact that, in *X. laevis* oocytes, the nuclear hY1 RNA indeed is associated with the La protein, whereas after or during export to the cytoplasm, this association is lost (Simons et al., 1994). The same phenomenon of La dissociation during nuclear export was observed previously in the case of 5S rRNA (Guddat et al., 1990) and SRP RNA (He et al., 1994), and is probably caused by 3'-end processing events leading to deletion of the La binding site. Interestingly, characterization of the Ro60-associated Y RNAs from *X. laevis* showed that these lack a functional La binding site (O'Brien et al., 1993). In this respect, the *X. laevis* Y RNAs differ from the human Y RNAs, for which it has been demonstrated that they are associated stably with the La protein in the cytoplasm as well (Boire & Craft, 1990; Peek et al., 1993). This implies that, in human cells, the Y RNAs either reassociate with cytoplasmic La protein after transport, or that in these cells the Y RNAs are transported in a complex containing the La protein.

As a consequence of the inability to associate with the La protein, hY1Xba RNA is exported at a much higher rate than hY1wt RNA. This can be explained by assuming that association of La to hY1wt RNA leads to retention of the RNA in the nucleus, whereas the hY1Xba RNA missing the La binding site is not retained and is exported immediately. An alternative explanation could be that, due to the mutation, the structure of hY1Xba RNA is changed in such a way that a binding site for a tRNA-specific export factor is created, resulting in fast nuclear export. However, this possibility seems unlikely because (1) only two terminal nucleotides at the 3' end, which are known not to be involved in secondary structural elements (Van Gelder et al., 1994), were changed in the hY1Xba RNA, and (2) hY1wt RNA appeared to compete for hY1Xba RNA export equally well as the hY1Xba RNA mutant (data not shown). In good agreement with the fast nuclear export of RNA mutants lacking the La binding site, we could show that La itself is very efficiently retained in *X. laevis* nuclei (Simons et al., 1996). Furthermore, it has been shown that La is one of the factors that is involved in the nuclear retention of U6 snRNA (Boelens et al., 1995).

It has been shown that hY1wt RNA associates with the La protein after injection into the nucleus (Simons

et al., 1994), which probably leads to its nuclear retention. Subsequently, the 3' end of the hY1wt RNA molecule is shortened gradually, a process that might be dependent on the dissociation of the La protein, allowing rapid export of the RNA to the cytoplasm. Thus, the nuclear export rate of hY1wt RNA in oocytes is in fact dominated by two counteracting processes: retention of the RNA mediated by La binding, and active export requiring the association with Ro60.

Mutant hY1Xba RNA is exported at a very high rate: within 1 h, it is completely exported to the cytoplasm. So far, the only RNA that was observed to be exported as fast as hY1Xba RNA is tRNA$_i^{met}$ (Zasloff, 1983; Jarmolowski et al., 1994), which is also an RNA polymerase III product. Similar to hY1Xba RNA, the tRNA molecule analyzed in these studies also lacked a functional La binding site. The relatively low export rates reported for SRP RNA (He et al., 1994) and 5S rRNA (Guddat et al., 1990; Jarmolowski et al., 1994), two other RNA polymerase III products, might also be explained by retention in the nucleus via binding to La followed by export after release of the La protein. These results imply that La is not required for nuclear export of RNA polymerase III products and that in *X. laevis* oocytes, the export rate of RNA polymerase III transcripts is strongly influenced by dissociation from the La protein and/or 3'-end trimming to disrupt the La binding site.

hY1 RNA export is saturable and requires a factor which is also needed for tRNA export

As discussed before, Ro60 binding seems to be a prerequisite for Y RNA to leave the nucleus. As a consequence, Y RNA export is expected to be a saturable process and indeed co-injection of increasing amounts of unlabeled Y RNA interfered with export of radiolabeled Y RNA. This effect is not necessarily due to a potentially limiting amount of Ro60, but may involve other, possibly more general export factors. Therefore, co-injections with other types of RNA were performed as well.

Cross-competition experiments in which large amounts of either tRNA$_i^{met}$, U1ΔSm RNA, 5S rRNA, or U6Δss RNA were co-injected with hY1Xba RNA, showed that hY1 RNA export could only be blocked with high concentrations of tRNA$_i^{met}$. This indicates that at least one *trans*-acting factor involved in the export of hY1 RNA is also involved in the export of tRNA$_i^{met}$. This factor is most likely different from Ro60, because Ro60 does not interact with tRNAs (Hendrick et al., 1981).

Although two other RNA polymerase III transcripts, 5S rRNA and U6 snRNA, did not interfere with the export of either hY1 RNA or tRNA$_i^{met}$, the present results do not exclude the possibility that this factor is involved in export of other RNA polymerase III prod-

ucts as well. Both 5S rRNA and U6Δss RNA were not exported detectably in the time period of the experiment and their inefficiency/inability to enter the export pathway may explain their inert behavior toward Y RNA or tRNA export. Further studies are required to investigate whether this factor is also involved in the export of other RNAs. It is likely that this factor has a higher affinity for human tRNA$_i^{met}$ as compared to human Y1Xba RNA, because a larger amount of hY1Xba RNA is necessary to saturate tRNA$_i^{met}$ export than the amount of tRNA$_i^{met}$ required to saturate the hY1Xba RNA export. It will be interesting to identify and characterize this export-mediating factor.

hY1 RNA export is not dependent on a Rev-like NES

The recent identification of nuclear export signals, small sequence elements rich in leucine and other hydrophobic amino acids (Gerace, 1995), prompted us to investigate whether export of hY1 RNA was also mediated by such a signal. Co-injection studies with a Rev peptide containing a nuclear export signal that is also involved in export of U1 snRNA showed that, although export of U1ΔSm RNA was blocked specifically, the export of both tRNA$_i^{met}$ and hY1Xba RNA was not influenced. This suggests strongly that Ro60 and the yet unidentified *trans*-acting factor(s) involved in the export process of hY1 RNA, do not contain a Rev-like nuclear export signal. The question remains whether Ro60 and/or the other factor(s) contain a different type of export signal or whether their mode of action in the export process of hY1 RNA is different.

MATERIALS AND METHODS

cDNA constructs

The transcription vector for hY1wt RNA has been described by Simons et al. (1994). The mutant hY1 RNA constructs were made from this hY1wt RNA construct by PCR techniques. The PCR products were cloned into pUC18. *Xenopus* U1ΔSm RNA and 5S rRNA constructs and human tRNA$_i^{met}$ and U6Δss RNA constructs with promoters for transcription by T7 RNA polymerase have been described previously (Hamm et al., 1987; Hamm & Mattaj, 1989; Chow et al., 1992; Jarmolowski et al., 1994). For transcription, the clones were linearized with *Dra* I (hY1wt, hY1ΔC, hY1ΔS1L1, U6Δss, and 5S), *Xba* I (hY1Xba and hY1XbaΔC), *Bam*H I (U1ΔSm), or *Bfa* I (tRNA$_i^{met}$).

In vitro transcription

For ^{32}P-labeled RNAs, 0.5 μg of linearized template DNA was transcribed in 10 μL buffer containing 40 mM Tris-HCl, pH 7.9, 6 mM MgCl$_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.1 mg/mL BSA, 1 mM NTPs, 2 U RNasin, 1 mM m7G(5')ppp(5')G (in case of U1ΔSm RNA), and 20 μCi

α^{32}P-UTP with 10 U of T7 RNA polymerase. Unincorporated nucleotides were removed by gel filtration and proteins were removed by phenol-chloroform (1:1) extraction. Nonradioactive competitor RNAs were prepared as described by Jarmolowski et al. (1994).

Oocyte injection

The RNA was microinjected into the nucleus of *X. laevis* oocytes (Simons et al., 1994). To control nuclear injection, samples were mixed with dextran blue (Serva Biochemicals) (10 mg/mL) and a total volume of 25 nL was injected per nucleus. After manual dissection, only oocytes with blue nuclei were used for analyses. After incubation and dissection, the fractions were homogenized in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1.5% SDS, 300 mM NaCl, 1.5 mg/mL proteinase K, and incubated at 56 °C for 30 min. Proteins were extracted with phenol/chloroform (1:1) and RNA was precipitated by adding 4 volumes of ethanol and analyzed on a 10% denaturing polyacrylamide gel. BSA-R and BSA-M have been described by Fischer et al. (1995) and 250 ng of each was co-injected per oocyte.

Immunoprecipitation

Antisera were coupled to protein A-agarose by head over head rotation at room temperature for 1 h in IPP500 (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% NP-40). For each precipitation, 50 μL of monoclonal anti-La (SW5) (Pruijn et al., 1995) or anti-Ro60 (2G10) (Veldhoven et al., 1995) or 1 μL normal human serum were used. Ten microliters of HeLa S100 extract (108 cells/mL) were mixed with ^{32}P-labeled RNAs and incubated for 30 min at 0 °C. Immunoprecipitations were performed in IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MgCl$_2$, 0.1% NP-40) by head over head rotation for 2 h at 4 °C. The precipitate was washed three times with IPP150 and the RNA was extracted and analyzed as described in the oocyte injection section.

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